SRSFC Project Progress Report (2014-17)

Proposal Category: Research **Proposal Status: Continuing Project**

Proposal title: Species identification and examining QoI and Captan resistance among *Colletotrichum acutatum* and *C. gloeosporioides* isolates found in VA vineyards.

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OBJECTIVES:

1. Determine whether *Colletotrichum* isolates from VA vineyards exhibits either QoI and/or caption resistance using a combination of *in vitro* and molecular approaches.
2. Identification of species among our *Colletotrichum* isolates using a targeted sequencing

In 2012-14 seasons, our lab collected over 450 *Colletotrichum* isolates from VA vineyards. To our surprise, about 50% seems to be *C. gloeosporioides*, based on colony and spore morphologies. Since it is often assumed *C. acutatum* is a predominant species in vineyards, more than the expected number of *C. gloeosporioides* in our sample maybe the artifact of consecutive captan applications. However, our species identification methods are somewhat subjective, considering number of studies revealed that both *C. acutatum* and *C. gloeosporioides* are composed of multiple species, rather than just single species [1-6]. Our culture collections also show wide variety of colony as well as spore morphologies.

In the 2014 and 2015 proposals, we are requesting support to investigate 1) potential QoI and/or captan resistance and 2) species identification of our collection of *Colletotrichum* species. Since ripe rot will affect grape yield and wine quality, the proposed objectives meet research priorities suggested by the VA Vineyard Association's annual survey (impact on fruit/wine flavor is listed #1 on both viticulture and enology section). The proposed research also meets research needs criteria of National Grape and Wine Initiative's Vision 2012 (the research priority 1: understanding and improving quality and 4: extension/education), as well as that of the National Road Map for Integrated Pest Management. Moreover, since both *C. acutatum* and *C. gloeosporioides* cause important diseases on strawberry and apple, our results can be applied to other regions of Southern US.

Materials and Methods

Isolate collection (completed in 2013-2014): In order to identify potential regional differences, growers were selected randomly from each of five major grape growing regions of Virginia (VA). The northern region spans from the Washington D.C./Arlington area west towards the Appalachian Mountains and south to the edge of the Monticello American Viticultural Area, which is the start of the central region that spans as far east as Richmond, VA. The western region of VA contains the Appalachian and Blue Ridge Mountain areas while the eastern region of VA spans the coastline from Virginia Beach up to the Chesapeake Bay area. The southern region of VA is the southern piedmont of Appalachia. Our lab visited 35 growers in 2013 and 7 in 2014 to collect symptomatic ripe rot grape berries. Since some growers had more than one vineyard, we collected from 90 unique locations, and within the same location we were able to collect some isolates. Thus, overall, we processed more than 1,000 samples.

In the field, the berry samples were sealed in a plastic bag and stored in a cooler containing ice. In the lab, skin from the berry was cut into approximately 5 x 5 mm piece, and the tissue was surface sterilized using 70% EtOH for 10 sec, followed by 0.53% hypochlorite (i.e. 10% bleach) solution for 1.5 min, and rinsing 1.5 min with autoclaved H_2O. Then the tissue was placed onto ¼ PDA amended with streptomycin and chloramphicol (both are 100 mg/ml). Once colony growth was observed, a hyphal tip was transferred onto a new Antibiotic ¼ PDA.

Once a clean colony was established, single spore isolations were made. Five- to seven-day old culture was flooded with autoclaved H_2O, and the media surface was gently scraped with a sterilized toothpick. Then the spore suspension was filtered with two layers of Miracloth (EMD Millipore) to remove mycelium. The spore suspension was streaked out onto a water agar in much the same way we would with bacteria to get individual colonies, and then individual spore or a hypha coming from individual germinated spore after 12-24 hours incubating was picked for further culturing using a sterile needle under microscope. The long-term storage of isolates were made by keeping dried fungal agar fragments and glycerol stocks **(**mycelium grown on a liquid media into 20% glycerol solution) at -80°C.

As of October 2014, we are estimating 450 of our collections were either *C. acutatum* or *C. gloeosporioides* based on colony morphology and spore characteristics.

Validation of G143A mutation in samples

DNA Extraction: Mass of mycelium from each culture plate will be placed into grinding bags (BIOREBA, Switzerland) containing 2 ml of a filter-sterilized grapevine extraction buffer (1.59 g/liter Na_2CO_3, 2.93 g/liter $NaHCO_3$, 2% Polyvinylpyrrolidone-40, 0.2% Bovine Serum Albumin, and 0.05% Tween 20) (Sigma-Aldrich Co. LLC, St. Louis, MO), and will be gently ground using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]). (It seems that the grinding is not necessary, but we are still tweaking the methodology.) Crude extracts will be then transferred into 1.5-ml microcentrifuge tubes and stored at -80°C until the next step.

PCR: To amplify pathogen cytochrome *b* gene fragments from total DNA, the following PCR primers [7, 8] will be used: RSCBF1, 5′-TATTATGAGAGATGTAAATAATGG-3′ and RSCBR2, 5′-AACAATATCTTGTCCAATTCATGG-3′. PCRs were performed in a total volume of 25 µL [12.5 µl GoTaq Green Master Mix (Promega) (2X), 1µL of each primer (10 µM), 3 µL of genomic DNA, and 7.5 µL H_2O]. PCR conditions will be 3 min at 94°C, followed by 40 cycles of 0.5 min at 94°C, 1 min at 54°C, 1.5 min at 72°C, a final extension for 8.5 min at 72°C, and holding at 4°C. PCR products will be separated by electrophoresis on a 1.5% agarose gel in 40 mM Tris- acetate (pH 8.0) and 1 mM EDTA (TAE) buffer and stained with ethidium bromide. In addition, to confirm G143A mutation, PCR products were sequenced at the University of Kentucky Advanced Genetic Technologies Center. Samples from 2014 collection will be tested.

Species identification

Based on the observations on colony morphologies among isolates from different locations (grower/vineyard/sample site) and crops, we are estimating to have approximately 304 unique isolates in our samples. Using the extracted DNA (culturing of isolates and DNA extraction methods were described above.), following methods will be performed to identify species. A species-specific internal transcribed spacer region 1 (ITS1) primer and the conserved universal primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') encoded in the 28S ribosomal subunit were used in pairs to identify isolates to species [9]. The ITS1 primers used were either the *C. gloeosporioides* specific ITS primer 5'-GACCCTCCCGGCCTCCCGCC-3' or the *C. acutatum* specific ITS primer 5'-GGGGAAGCCTCTCGCGG-3' [2, 10]. Isolates were assigned to the species group by which a positive amplification with a specific ITS1 primer was obtained. The PCRs were performed in a total volume of 25 µL [12.5 µl GoTaq Green Master Mix (Promega) (2X), 1 µl species-specific primer (ITS1) (10µM), 1 µl conserved universal primer (ITS4) (10µM), 2 µl DNA, and 8.5 µl H_2O]. The PCR conditions for the *C. gloeosporioides* specific/ITS4 pair were 5 min at 94 °C, then 26 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 2min, and then 7 min at 72 °C. The PCR conditions for the *C. acutatum* specific/ITS4 pair were 5 min at 94 °C, then 32 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 2min, and then 7 min at 72 °C. The resulting amplification products were verified by electrophoresis described above.

To confirm species identification, PCR products were sequenced directly in both directions at the University of Kentucky Advanced Genetic Technologies Center. Sequences were trimmed and aligned using Lasergene 9.1 software. Each consensus sequence served as an individual BLAST query against NCBI GenBank and Q-bank databases. In addition, BLAST queries will be coupled with phylogenetic analyses leveraging maximum likelihood and Bayesian methods, to identify species of *Colletotrichum* in our collections. Colony morphology of the each culture on ¼ PDA, as well as spore size and characteristics were recorded to aid our species identification.

Results

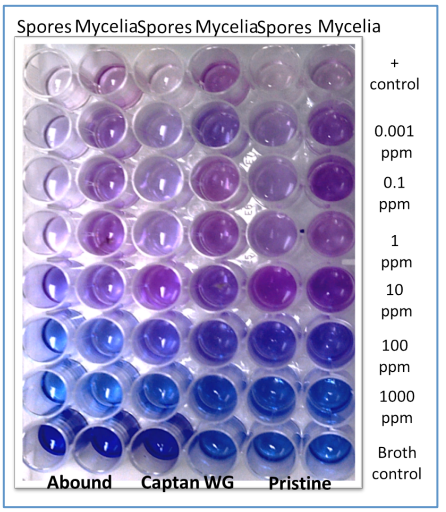
Fungicide sensitivity tests

We have been using two-step methods to reduce costs. The first step is use of Alamar blue (AB) in the fungicide amended culture plate [11], and then we will use more traditional fungicide amended media with potentially resistant isolates.

In order to develop a protocol for *Colletotrichum* species, we have conducted a preliminary study with AB assay to screen several mode of action groups using two of our isolates. At the beginning of the assay, we had some concern on the proposed filtering (which was adapted from the original paper (15)) since some of fungicides, such as Abound, left quite a bit of residues on the surface of the filter. We were not certain that we could consistently achieve the correct end concentration after the filtering. In addition, the original paper used V8 as a medium, but we learned that it is very difficult to filter V8, and also it was difficult to obtain consistency with V8 media. After several trials, we made several modifications to the protocol to make it work with our isolates. We changed medium from V8 to 2% PDA and fungicide stocks were prepared with ethanol to reduce contamination.

Modified protocol: The single-spore isolate plates will be flooded by adding 5 ml of clarified, buffered 2% Potato Dextrose Broth (PDB). Then, the suspension will be filtered using two layers of Miracloth to remove mycelium. Then, 100 µl of a suspension of 10^5 conidia/ml (adjusted using a hemocytometer) or 100 µl of 2% PDB with aerial mycelium will be added to test wells of 48-well cell culture plate (Corning Costar), and stock fungicide solutions will be added to give final concentrations of each fungicide (0.0, 0.01, 0.1, 1.0, 10.0, and 100.0 µg/ml Note: for some of fungicides, rate up to 500 ppm were tested). AB dye (AbD Serotec) will be added as 10% of the final volume in the test wells [12]. Plates will be covered with sterile plastic plate covers, gently rotated horizontally to mix the well contents, then incubated in the dark at 25°C for 48 h. There will be negative control (200 µl of 2% PDB and 10% AB dye only), and positive control (100 µl of PDB, 100 µl of 10^5 conidia/ml, and 10% AB dye). A chemical control plate will be also prepared to ensure that the fungicides themselves did not reduce the AB dye (100 µl of stock fungicide, 100 µl of 2% PDB, and 10% AB).

Figure 1
AB plate test example from our test



A positive test result was recorded as a color change from blue to pink, which indicated that the dye had been reduced due to the presence of viable conidia (Fig 1). A negative test result will be recorded as no color change or the dye remained blue, i.e., the dye was not reduced due to the absence of viable conidia/fungal growth. One mean inhibitory concentration (MIC) endpoint will be visually determined and defined as the lowest concentration of fungicide that prevented a color change from blue to pink (MIC- blue) after 48 h of incubation. Optimal incubation time was determined by monitoring the color of the negative control wells, and set to 48 hours.

Table 1 shows the results from our preliminary experiments. EC50 (Effective concentration with 50% inhibition) was determined with a logistic regression in JPM 10Pro (SAS, Cary, NC). Based on the assay, we did not find significant difference ($P<0.05$) between two isolates (*C. acutatum* and *C. gloeosporioides*), and many of fungicide resulted in less than field rate EC50. However, one of fungicides that showed an excellent efficacy with our table grape assay (*data not shown*), Mettle, did not work with AB assay. At the same time, we had to use a buffer (sodium bicarbonate) to increase the pH of Mettle treated well, so, it might have affected its efficacy. As with any other *in vitro* tests, tests using a live plant tissue should be conducted to validate true efficacy. The poor performance by Endura was also shown with our table grape assay, thus, SDHI group probably does not have efficacy against *Colletotrichum* acutatum and *C. gloeosporioides*.

Table 1. Estimated EC50 values for each fungicide based on AB assay: Bold = exceeded the field ppm, '*' = different' formulation of copper, '**' median rate were used when applicable

					C. acutatum		*C. gloeosporioides*	
Trt #	Fungicide	Chemical name	FRAC code	PPM in field**	Mycelia	Spores	Mycelia	Spores
1	Abound	azoxystrobin	11 (QoI)	215.3	34.4	187.0	46.9	205.3
2	Captan 80	captan	M4	1922.2	103.8	40.7	59.4	30.2
3	Dithane	mancozeb	M3	1333.5	36.0	10.0	20.9	363.8
4	Topsin M	thiophanate-methyl	1 (Beta-tublin inhibitor)	841.0	215.2	143.6	257.8	210.1
5	Mettle	tetraconazole	3 (DMI)	36.0	24.3	**37.8**	**69.3**	**67.6**
6	Endura	boscalid	7 (SDHI)	420.5	**701.8**	**445.6**	**434.3**	**494.0**
7	Prophyt	phosphorous acid	33	3382.9	129.2	85.1	139.8	63.8
8	Champ	fixed copper	M1	2775.2	147.5	76.1	187.5	131.9
8*	Cueva	fixed copper	M1	2000.0	75.0	50.0	75.0	125.0
9	IKF-309	unknown	U8 (Actin Disruptor)	70.3	**316.4**	**377.9**	**301.9**	**494.0**

Once some of isolates are identified to be less sensitive to the fungicide, traditional mycelium growth and spore germination tests will be conducted to confirm their lack of sensitivity to fungicides. Plates of ¼ PDA will be amended with stock fungicide solution to give final concentrations of 0.0, 0.01, 0.1, 1.0, 10.0, 100.0, and 1,000.0 µg/ml for each fungicide + SHAM (100 µg/ml).

Mycelium growth: A 5 mm diameter agar block will be cut from the advancing edge of an actively growing culture on ¼ PDA (using a 100 mm Petri dish) and placed in the center of the dish, mycelia-side down, on the surface of the amended PDA. Plates will be incubated in the dark at 25°C for 4-6 days. The radial diameter (perpendicular measurements in millimeters) will be recorded for each colony. The corrected diameter (mean radial diameter minus the length of the agar block) will be used to calculate percent relative growth (%RG = [mean diameter of colony/mean diameter of colony on non-amended agar] × 100) and percent relative growth inhibition (%RGI = 100 – %RG) compared with the non-amended controls. At each experiment, three plates will be used per isolate, the experiment will be conducted twice, and the mean corrected colony diameters will be used in all calculations.

Germination rate: Four 5 µl of spore suspension with 1 x 10^6 spore/ml will be placed on to aforementioned amended (and non-amended) ¼ PDA. Then spore germination rate (and formation of appressorium) will be determined using microscope (40x and 100x objectives, Nikon Eclipse Ci, Nikon, Inc.). The observation will be made at 6 hours after inoculation, and 25 spores will be examined per drop (i.e., 100 spores will be examined per isolate per run). Percent germination and relative germination inhibition rate will be determined. The assays will be conducted three times. Data from mycelium growth and spore germination rate will be analyzed using linear (PROC REG) or non-linear (PROC NLIN) or other methods such as beta model [13] regression to determine EC_{50} (Effective Concentration to inhibit 50% of sample) and EC_{90}. In 2014, 36 isolates were selected based on their genetic similarities (please see the section below), host (some are from apples and strawberries), and geographic locations.

A first run of mycelium growth test has been conducted in Oct-Nov. 2014 using four isolates selected based on the G143A mutation test (described below). Due to contamination issues, which seem to be attributed to our fungicide stock, we may need to repeat the test. Currently we are in the middle of data collection and analysis from the first set of experiments. Germination rate tests will be conducted once we figure out the source of contamination.

G143A mutation in samples: We completed all of 2013 isolates, got 292 *Colletotrichum* isolates (99, Ca and 193, Cg). Since high levels of resistance are indicative of a target site mutation, we sought to characterize the cytochrome b gene to determine if there were mutations that were specific to the resistant isolates. Amplification of an internal portion of the cytb gene using primers RSCBF1 and RSCBR2 produced a single fragment, of ≈285 bp in size. Cloned cytb gene fragments derived from a QoI-resistant and -sensitive isolate of grape ripe rot. Positive PCR-product bands were obtained from all *C. acutatum* isolates (99/99), but only 36 from 193 *C. gloeosporioides* isolates. The PCR product then need to be treated using a mutation-specific restriction enzyme

ItaI (=Fnu4HI). The PCR products from resistant isolates carrying the mutated sequence GCN at position 143 could be digested by the restriction enzyme, whereas those from sensitive isolates remained undigested.

Species identification: In 2014, PCR products (ITS gene) of all of 2013 samples (368 isolates, which was more than we expected) were sent out for sequencing. Two complementary approaches were used to assign the *Colletotrichum* isolates to previously characterized *Colletotrichum* species. The first approach queried the isolated *Colletotrichum* sequences against both the GenBank and Q-bank databases. The top BLAST pairwise hits were used to identify the closest *Colletotrichum* species. Based on BLAST searches from GenBank and Q-bank databases, ITS sequence data showed that a total of 304 isolates have been accepted as *Colletotrichum* species.

Pairwise DNA similarity matches are effective at identifying previously characterized genes; however, they do not reveal the inferred evolutionary relationships between the assigned *Colletotrichum* species. Therefore, the second approach, phylogenetic analyses were also performed to understand the phylogenetic relationships between the *Colletotrichum* isolates and vouchered reference *Colletotrichum* species (31 species within the *C. acutatum* complex (Damm et al., 2012) and 22 species plus one subspecies within the *C. gloeosporioides* complex (Weir et al., 2012)). We are still in the process of analysis, especially for some isolates that didn't fit into the grouping, but it seems that we identified at least six distinct species group in *C. acutatum* and eighteen *C. gloeosporioides* groups. At the same time, the phylogenetic analyses indicated that the ITS gene gave a low resolution and poor bootstrap support gene tree, which seemed to have less phylogenetic signal. The results we found from ITS sequences data was consistent with the previous research demonstrating many of the species are unable to be reliably distinguished using ITS, the official barcoding gene for fungi. Particularly problematic are recent study showed that *C. acutatum* and *C. gloeosporioides* are actually two species complex with potentially 20+ species for each complex.

The phylogenetic results from Maximum Likelihood (ML) analysis showed overall poor support for the relative branch order within ITS region. Sequences for this phylogenetic analyses included voucher *Colletotrichum* species (31 species within the *C. acutatum* complex and 22 species plus one subspecies within the *C. gloeosporioides* complex), two fungal outgroups (*Colletotrichum fragariae*_KC411911 and *Alternaria alternata*_HQ846574) (We randomly chose a fungal species, that belong to the same family as *Colletotrichum*, *Alternaria*, but represent different genera. And also these outgroups were identified using the same single DNA sequence gene, ITS.), and some unknown isolates from different species groups (six distinct species group in *C. acutatum* and eighteen *C. gloeosporioides* groups) (Fig. 2 and 3).

The selected isolates from different species groups were not clustered into reference *Colletotrichum* species. Given the overall poorly supported topology in ITS region it was not possible to confidently assign our isolates to any one of the reference *Colletotrichum* species within the *C. acutatum* and *C. gloeosporioides* complex based on the ITS region alone. We have obtained sequence data from a second gene (TUB2 for Ca and CAL for Cg) as combination genes, which would give more phylogenetic signal for species identifications.

Figure 2 A phylogenetic tree based on ITS regions of six isolates from different species groups, 31 species within the *C. acutatum* complex as reference *Colletotrichum* species, and two fungal outgroups.

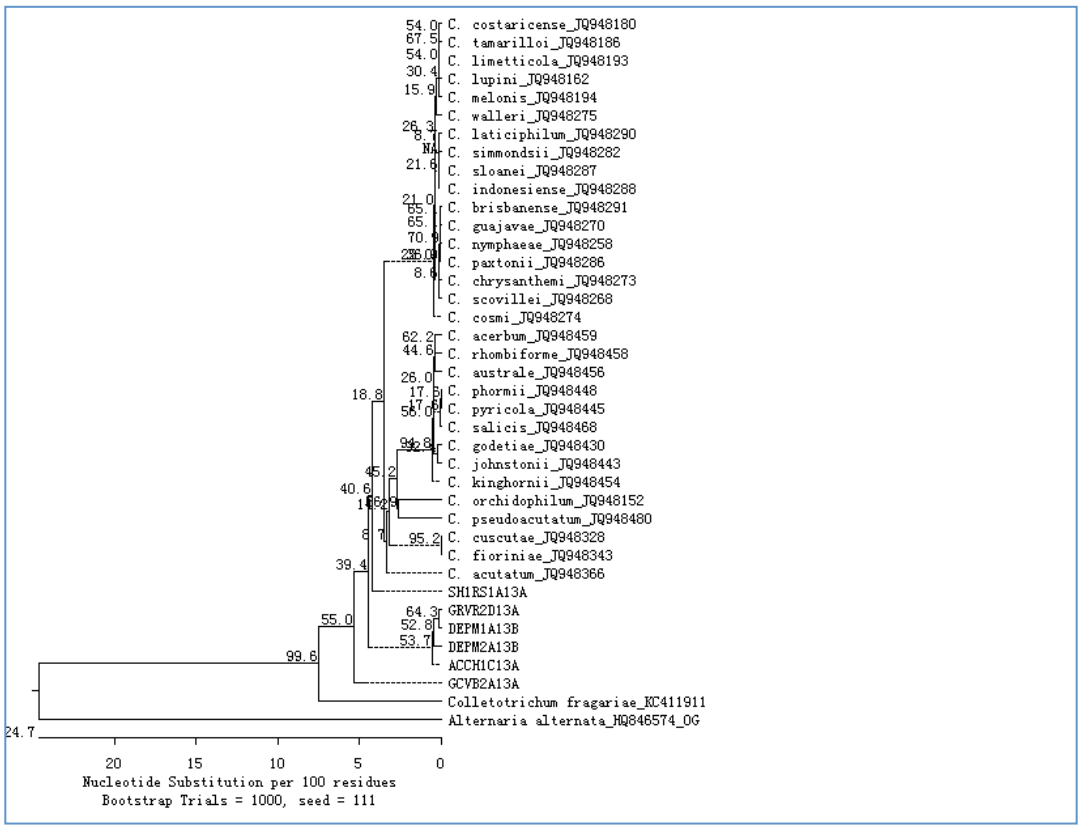
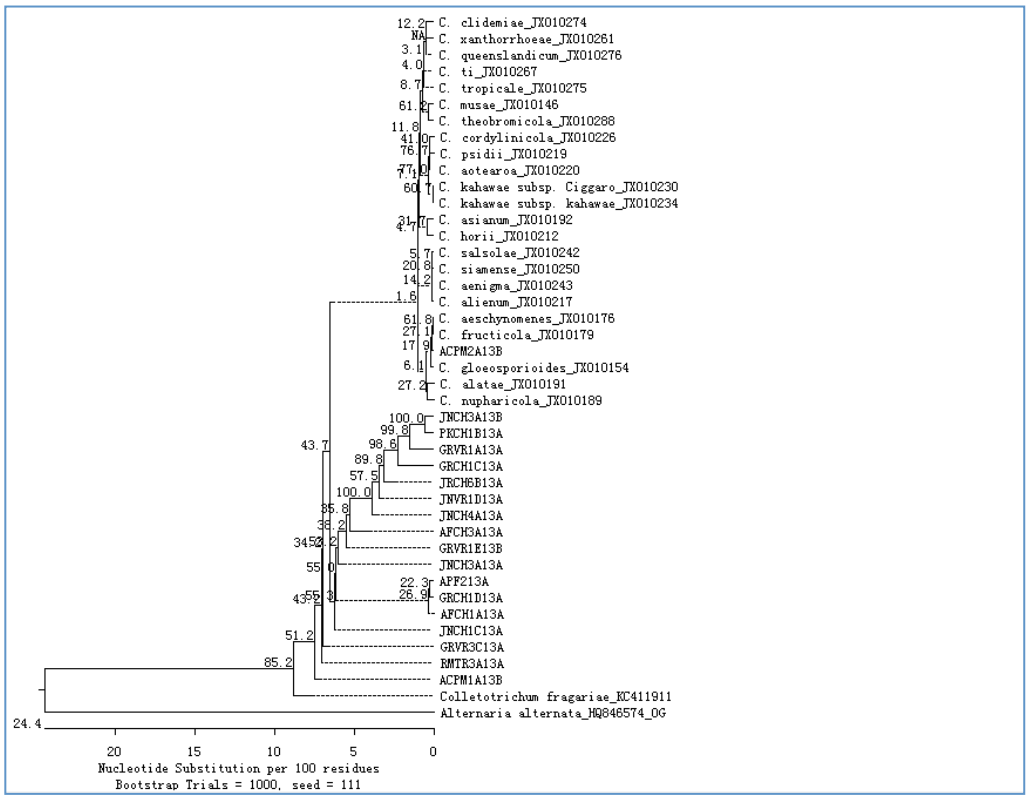


Figure 3. A phylogenetic tree based on ITS regions of 18 isolates from different species groups, 22 species plus one subspecies within the *C. gloeosporioides* complex as reference *Colletotrichum* species, and two fungal outgroups.



Future direction:

1) Continuation of AB assays and plate assays using other isolates we obtained through the survey
2) Continue to identify *Colletotrichum* to the level of species based on the single genes and combinations of genes analyses, will do both separate phylogenies for the first and second genes (combining our data and the previously published data for each gene) and then a third partitioned dataset that examines both genes in a single phylogenetic analysis.
3) Based on the species identification study, we will choose candidates from different species within the *C. acutatum* and *C. gloeosporioides* complex combined with sampling information, such as location, variety, number of QoI-based sprays if possible, and conduct a bioassay using fungicide amended media to determine the level of resistance. In addition, we are also planning to conduct a bioassay to screen for captan resistance.

Expected outcomes/ Potential impact from this study

Based on wide spread usages of QoI fungicides among VA vineyards, as well as previous records on QoI resistance in both *C. acutatum* and *C. gloeosporioides* [8], we think there is a good chance that we will find some QoI insensitive isolates from our samples. We are also expecting to identify a few different species (or subspecies) in our samples due to wide variety of colony morphologies, variations in ITS regions, as well as currently on-going species complex discussion on both species [1-6]. The results will be discussed in extension meeting in and out of VA, and reflected on extension publications. We are planning to publish our results on scientific journals at the end of the project.

We believe the impact of this study will be very high, not only because of the increasing acreages of vineyards in Southeastern US, but also because these pathogens can cause diseases on other crops grown in the region (e.g., strawberry anthracnose, apple bitter rot). Also, species ID and fungicide resistance study will give us an insight on how to manage this economically important disease.

Presentation made in 2014

Nita, M., Hartely, S., and Oliver, C. "Screening of Fungicides for the Control of Ripe Rot on Grapes" American Phytopathological Society National Meeting, 5 Aug. 2014

Oliver, C. and Nita, M. "Characterizing the infection conditions of grape ripe rot (*Colletotrichum acutatum* and *Colletotrichum gloeosporioides)* on wine grape clusters", American Phytopathological Society Potomac Division meeting, 13 March 2014

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